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## Research Paper

## Microplastics released from food containers can suppress lysosomal activity in mouse macrophages

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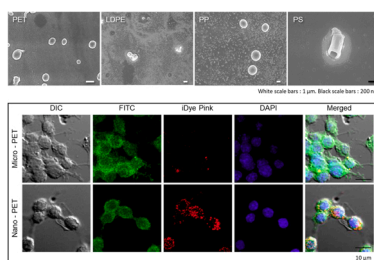
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## HIGHLIGHTS

- Food packaging usage represents a large proportion of rising demands during the COVID-19 pandemic.
- High-resolution analysis detects large amounts of micro and nano plastics leachates from plastic food packaging.
- The reuse of plastic food packaging, including PET and PP, brought the continual release of particles.
- Microplastics could accumulate in cells and suppress activities of lysosomal with long-term effects on the human.

## GRAPHICAL ABSTRACT



## ARTICLE INFO

Editor: Youn-Joo An

## Keywords:

Plastic food packages  
Microplastics  
Nanoplastics  
Macrophage activity

## ABSTRACT

The ingestion and accumulation of microplastics is a serious threat to the health and survival of humans and other organisms given the increasing use of daily-use plastic products, especially during the COVID-19 pandemic. However, whether direct microplastic contamination from plastic packaging is a threat to human health remains unclear. We analyzed the market demand for plastic packaging in Asia-Pacific, North America, and Europe and identified the commonly used plastic food packaging products. We found that food containers exposed to high-temperature released more than 10 million microplastics per mL in water. Recycled plastic food packaging was demonstrated to continuously leach micro- and nanoplastics. In vitro cell engulfing experiments revealed that both micro- and nanoplastic leachates are readily taken up by murine macrophages without any preconditioning,

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and that short-term microplastic exposure may induce inflammation while exposure to nanoplastic substantially suppressed the lysosomal activities of macrophages. We demonstrated that the ingestion of micro- and nanoplastics released from food containers can exert differential negative effects on macrophage activities, proving that the explosive growth in the use of plastic packaging can pose significant health risks to consumers.

## 1. Introduction

The escalating environmental pollution by microplastics (hereby defined as plastic particles of around 100 nm to 5 mm (Hernandez et al., 2017; Alimi et al., 2018)) is of grave concern to the scientific community, policymakers, and society in general (Hwang et al., 2019; Browne and Galloway, 2007). Apart from microplastics in personal care products (Hernandez et al., 2017; Browne and Galloway, 2007), plastics used for packaging are a significant contributor to microplastic pollution. Globally, polyethylene terephthalate (PET), polyethylene (PE), polypropylene (PP), and polystyrene (PS) are the synthetic polymers most used in plastic packaging, with the more developed regions of Asia-Pacific, North America, and Europe being the chief consumers (Fig. 1, Figs. S1–5) (BBC, 2020). In particular, a substantial proportion of plastic packaging (67% by weight in 2020) is used in the packaging of perishables, notably food and beverages (Fig. 1b) (BBC, 2020). The use of these plastics is expected to increase globally, driven partly by the increase in the penetration of e-commerce platforms and current pandemic situation required takeaway hot food and food delivery service. Micro and nanoplastics are mostly expected coming from these containers during storage and reuse.

The tendency of microplastics to be uptaken into biological systems via inconspicuous ingestion or adsorption (Mercogliano et al., 2020) means that microplastic pollution and eventual accumulation gradually threatens the health and survival of all organisms (Mercogliano et al., 2020). Moreover, microplastics are potential mediums of exposure to chemicals (e.g., bisphenol and styrene) that have known adverse health effects (Chen et al., 2017; Hahladakis et al., 2018). Most relevant studies have focused on the ingestion of microplastics by aquatic organisms and have theoretically extrapolated that these ingested microplastics eventually reached the human gut through the food chain (Ivar do Sul and Costa, 2014). Recently, translocation and the associated accumulation of microplastics in mammalian cells have been closely linked to cytotoxicity induced chronic inflammation and negative oxidative stress resulted carcinogenesis in human (Banerjee and Shelper, 2021; Choi et al., 2020; Stock et al., 2021; Pérez-Albaladejo et al., 2020; Hwang et al., 2020). Such systemic infiltration of microplastics into the human body highlights the urgent need to identify and analyze the sources of plastic-derived contaminants. As studies on microplastic ingestion have focused on sources such as seafood (Ivar do Sul and Costa, 2014; Gangadoo et al., 2020), tap water (Kosuth et al., 2018), beverages (Shruti et al., 2021), teabags (Hernandez et al., 2019), PP water bottles (Mason et al., 2018), and PP infant feeding bottles (Li et al., 2020), whether plastic food packaging, which is ubiquitous and usually subjected to high temperature, contributes to microplastics contamination remains unclear. Previous studies focused on release of microplastics from heat-treated take-out food containers had yielded no significant leaching of particles. However, the analysis methods employed were for particles in the micrometer range (Du et al., 2020).

In this study, we hypothesized that plastic particles in the nanometer range are able to leach out of the plastic packaging. We employed highly sensitive and higher resolution analysis methodologies to demonstrate that plastic packaging exposed to high temperatures (e.g., food heated or cooked with its packaging, and packaging for hot beverages) do release a significant amount of plastic particles. We showed that repeated high-heat treatment of plastic food containers results in the continuous leaching of microplastics, thus contaminating the food packaged within and leading to direct human consumption. Microplastics released from such packaging were shown to be readily engulfed by RAW264.7 mouse

macrophages in vitro even without any prior conditioning. Our findings clarify the immediate negative implications of the use of plastics in food packaging. The current pandemic has undone many previous efforts to reduce plastic use, favoring disposability and hygiene over sustainability (Vanapalli et al., 2021), naturally leading to a drastic increase in the use of plastic products and packaging due to the need to spatially separate individuals. This further emphasizes the need for an alternative material that can effectively replace plastics and delay the projected “plastic pandemic” (Fadare and Okoffo, 2020; Klemeš et al., 2020).

## 2. Experimental section

### 2.1. Sample preparation and precautions

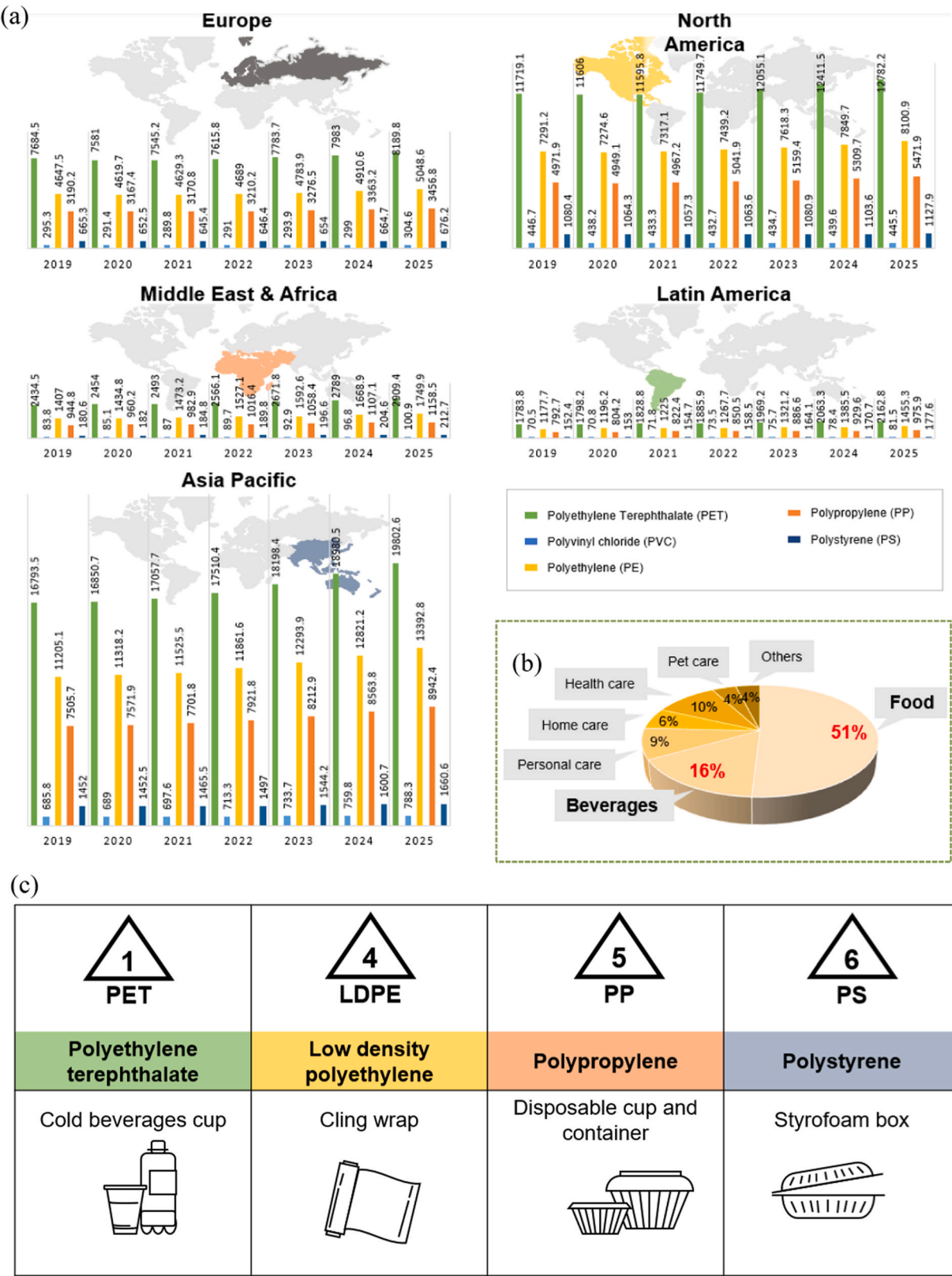
Four commercial plastic products, namely PET, LDPE, PP, and PS, commonly used for food packaging (as beverage cups, cling wrap, disposable cups, food containers, respectively), were purchased from grocery stores and coffee shops in Singapore. To prevent contamination by dust and debris, the plastic samples, glass vials with caps, and other hardware to be used in the experiments were thoroughly washed using DI water three times. To avoid contamination by other (non-sample) plastic particles, hardware that would come in contact with the samples was made from clean glass. A negative control sample (bare) was prepared by heating 25 mL of fresh DI water in glass vials for 10 min at 95 °C. The particle concentrations detectable in the bare samples were used as the baseline. These ambient particulate contaminations are inherent to the equipment, the atmosphere, and cleaned glass vials (leftover residues).

### 2.2. Release of particles from plastic food containers

The plastic products were cut with steel scissors into 2.5 cm × 2.5 cm samples. These samples were thoroughly washed three times with room-temperature DI water. It helped remove dust and plastic debris coming from cutting and subsequently dried in a clean petri dish in a desiccator cabinet. For each type of plastic examined in this study, four such plastic samples were immersed into a single glass vial containing 25 mL of DI water, heated at 95 °C for 10 min, and then removed for characterization. The water from the glass vial, which contained plastic leachate, was labeled as the water from the first heat cycle and cooled to room temperature for characterization. All experiments were performed in triplicate. The PS and PE samples were subjected to only one heat cycle, whereas the PP and PET samples were subjected to four heat cycles in new preheated glass vials, with the leachate collected and labeled after each cycle, and steeped for 10 min each time. The heat-treated plastic samples were washed with room-temperature DI water to remove plastic debris and dried in a desiccator cabinet at room temperature for characterization.

### 2.3. Dynamic light scattering (DLS) characterization

DLS (Zeta Potential Analyzer, Brookhaven Instruments, Gaia Science Pte. Ltd.) analysis was performed using disposable single sealed cuvettes (size: 50–2000 µL, Eppendorf) containing 200 µL of the leachate solution and BIC Particle Sizing software (Brookhaven Instruments). The effective diameter was detected in five measurement cycles (each cycle running for 1 min) at a scattering angle of 90 °. The light percent distribution and number percent distribution were also detected to verify and compare the particle size distribution.



**Fig. 1.** Global market demand for plastic packaging (BCC Research) (BBC, 2020). (a) Past and projected (2019–2025) use of plastic packaging worldwide. Polyethylene Terephthalate (PET), Polyethylene (PE), and Polypropylene (PP) account for the majority of the plastics used in the global plastic packaging market, which is dominated by consumers in the Asia-Pacific, North America and Europe. Unit: Kilotons. (b) Global revenue market share by end use (2020), showing that packaging for food and beverages contribute the most to plastics use. (c) Commonly used plastic food packaging and their ASTM D7611 symbols indicating their recyclability (Fick, 2018).

## 2.4. Field-emission scanning electron microscopy (FE-SEM)

The surface morphology of the plastic samples and the shape of the plastic particles were examined using FE-SEM. Untreated and treated plastic samples were washed with fresh DI water two times and dried in a desiccator box. To observe the plastic particles, glass slides (Sail Brand) were washed with acetone (Sigma) and DI water, treated with oxygen plasma (Plasma Cleaner PDC-002, Harrick Plasma) for 1 min, and then immersed in DI water for 2 min to increase its hydrophobicity. Thereafter, for each sample, 10  $\mu\text{L}$  of a solution containing the sample particles was dropped on the glass slides and dried. The dried samples and glass slides were immobilized on a sample holder with carbon tape and sputter-coated with a 5 nm thick platinum film using a JFC-1600 Auto Fine Coater (JEOL; operating settings: 30-mm distance, 20 mA, 40 s). FE-SEM imaging (JSM-7600F Schottky FE-SEM, JEOL) was performed at an accelerating voltage of 5.00 kV under various magnification levels between  $\times 5000$  and  $\times 200,000$  to capture particle in the nanometer scale.

## 2.5. Fourier-transform infrared (FTIR) spectroscopy

The plastic samples were washed with DI water and dried in a desiccator box before FTIR characterization on a PerkinElmer spectrometer (PerkinElmer) equipped with a diamond cell attenuated total reflection (ATR) accessory module. Reflectance infrared spectra were collected from  $4000\text{ cm}^{-1}$  to  $600\text{ cm}^{-1}$ , with 16 scans per measurement and three replicate measurements per sample. Background spectra were collected before sample readings and automatically subtracted from each measurement. Baseline correction and spectrum smoothing were performed using Spectrum 10 software (PerkinElmer).

## 2.6. Dynamic image particle analysis (DIPA)

The concentrations of plastic microparticles leached from the different samples were measured through DIPA using a benchtop Fluid Imaging FlowCAM system (Fluid Imaging Technologies) with a 100- $\mu\text{m}$  flow cell and a  $\times 20$  optical lens. The system was calibrated with 50  $\mu\text{m}$  polyester beads (Thermo Fisher Scientific). The flow chamber was washed with DI water before the measurements. Thereafter, 100  $\mu\text{L}$  of the liquid sample was injected into the flow cell using a syringe pump at a speed of  $0.05\text{ mL min}^{-1}$  for 2 min. The camera captured timelapse images (20 images per second) of the plastic microparticles. From these images, the particle concentration and distribution were estimated using Visual Spreadsheet software.

## 2.7. Confocal laser scanning microscopy (CLSM)

Water samples containing nanoplastics obtained from the steeping of PE samples dyed with iDye Pink were filtered through a 0.22  $\mu\text{m}$  syringe filter, following which clean DI water was filtered through the same syringe filter. The syringe filter unit was then carefully cut with a pair of shears, and the filter membrane inside was gently removed and mounted onto a glass coverslip. Fluorescence from the iDye Pink-labeled nanoplastics on the filter membrane was visualized on a LSM710 confocal microscope equipped with an Axio Observer Z1 inverted microscope with a 561 nm laser source and 572-622 nm filter.

## 2.8. Labelled microplastics for cell culture assays

Discs of respective plastic samples were dyed with iDye Pink (Jacquard Products) which is nontoxic, according to manufacturer's recommended method with some modifications. Briefly 14 g of iDye Pink powder was dissolved in a beaker of hot water, and the plastic samples were immersed for 2 h to allow a higher color intensity in the resultant plastic particles. The dyed plastic samples were then thoroughly washed in clean water until the water turns clear, before the plastic samples

were immersed into 70% ethanol solution and brought to a sterile biosafety cabinet. The plastic samples were then thoroughly washed with deionized water prefiltered using a 0.2  $\mu\text{m}$  Nalgene vacuum filtration system (Sigma-Aldrich) to remove any remaining ethanol. The respective plastic samples were then submerged in a petri dish with water and physical ground using 400 grit sandpaper to produce random-size plastic particles. The respective suspension of plastic particles was then filtered through Whatmann filter papers (6  $\mu\text{m}$  filter) to remove large sized particles. The filtrate was then run through a 0.45  $\mu\text{m}$  syringe filter. This filtrate was then run through a 0.22  $\mu\text{m}$  syringe filter. The retentate from the 0.45  $\mu\text{m}$  filter was recovered by carefully splitting the syringe filter unit and rinsing into a fresh vial of deionized water. Size characterization via DLS was performed for the retentate suspension and the filtrate from the 0.22  $\mu\text{m}$  filtration to represent microplastics in the micrometer range and nanometer range respectively. The suspensions were kept at  $4^\circ\text{C}$  until further use. Concentration of particles in each respective suspension was quantified via FlowCam (Fluid Imaging Technologies) using a 20X magnification lens.

## 2.9. Cell culture

RAW264.7 cells were cultured on poly-D-lysine (PDL, A3890401, Gibco)-treated 12 mm coverslips on 24 wells plates before treatment with 4  $\mu\text{g/mL}$  of  $191.6 (\pm 0.9)$  nm polyethylene terephthalate (PET),  $1.85 (\pm 1.02)$   $\mu\text{m}$  PET and low density polyethylene (LDPE), particles dyed with iDye Pink. Treated cells were incubated at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$  for 3–24 h before being fixed or stained for immunostaining or lysosomal/mitochondrial tracing.

## 2.10. Cell fixation and immunofluorescence staining

Cells were fixed with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) at  $4^\circ\text{C}$  for 16–20 minutes. Fixed cells were washed three times, at 5 min each, with PBS to remove excess PFA and permeabilized with 0.1% Triton X-100 containing PBS (PBS-Tx) for 15 min. Subsequently, the cells were blocked with 5% goat serum in PBS-Tx at room temperature for 1 h before incubation with the primary mouse anti-GAPDH antibody (1:1000; sc32233, Santa Cruz Biotechnology) diluted in 2.5% goat serum containing PBS overnight at  $4^\circ\text{C}$ . The next day, the cells were washed three times with PBS, for 5-min each, before the addition of Alexa Fluor 488 goat anti-mouse IgG (H+L) secondary antibody (1:1000, A-10680, Thermo Fisher Scientific) and 1:5000 dilution of 5 mg/mL 4,6-diamidino-2-phenylindole (DAPI) (Thermo Fisher Scientific, #D1306), prepared in 2.5% goat serum containing PBS, and incubation for 2 h at room temperature. The cells were washed three times with PBS for 5 min each to remove excess secondary antibodies before mounting on glass slides with Fluoromount Aqueous Mounting Medium (F4680, Sigma-Aldrich).

## 2.11. Lysosomal and mitochondrial tracing

For lysosomal and mitochondrial tracing, 200,000 cells were first seeded onto a 24-well plastic plate with a 12 mm glass coverslip overnight, before incubation with 4  $\mu\text{g/mL}$  of nano-PET and micro-PET particles for 3 h at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$ . After 3 h of incubation, either 1:20,000 LysoTrackerTM Green DND-26 (L7526, Thermo Fisher Scientific) or 100–400 nM MitoTrackerTM Green FM (M7514, Thermo Fisher Scientific) directly into the culture media for 15 min at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$ . After 15 mins of incubation, the culture media were replaced with DMEM in 0.5% fetal bovine serum (Life Technologies) and cells imaged live at  $40\times$  or  $100\times$  objectives through confocal microscopy.

## 2.12. Confocal and direct interference contrast (DIC) microscopy

All immunofluorescence and DIC images were acquired using a Nikon A1R HD25 confocal microscope and the Nikon DS-Qi2 camera

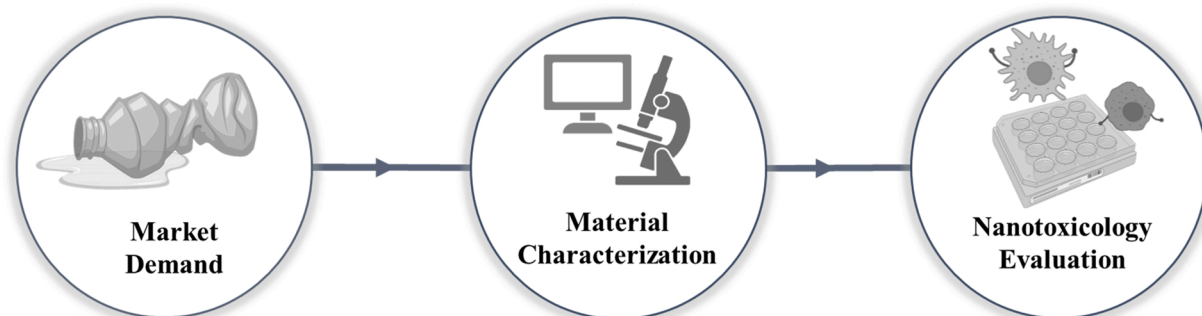
respectively at the Nikon Imaging Centre (NIC), Singapore. Images were captured at  $40\times$  objective. Z-series stacks of confocal images were generated with maximum intensity and resized to a resolution of  $1024\times 1024$  pixels. Merged images were obtained by cropping and aligning the confocal and DIC images using ImageJ software (Lima et al., 2016).

### 2.13. Lysosomal activity assay

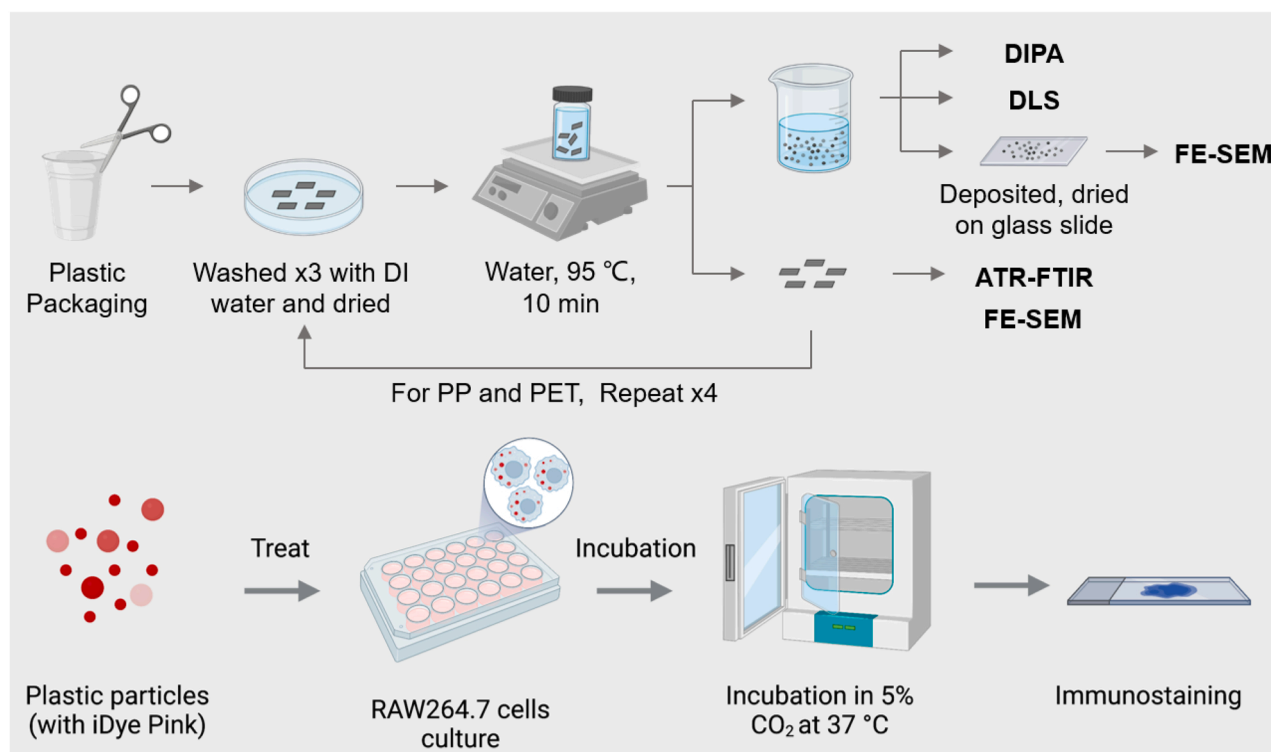
Lysosomal activity assay was performed following the manufacturer protocol (Cat: ab234622). Briefly, 500,000 cells were seeded onto a six-well plate and treated with micro-PET or nano-PET particles for 3 h at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$ . After which, a sub-quenched substrate was added to the culture medium and further incubated for 1 h. The medium was

washed twice with 1 mL of ice-cold assay buffer before live imaging under 488-nm filters through Nikon A1R microscopy. The same power intensity was maintained during imaging of all samples. Intensity density (IntDen) elicited from the quenching of the lysosomal activity was measured by manually tracing cells using the “Selection brush tool” and quantified using the “Measure” tool in ImageJ software (Lima et al., 2016). Pairwise comparison between untreated and PET-treated samples were performed using two-tailed Mann-Whitney test in GraphPad Prism 9.3.1.

(a)



(b)



**Fig. 2.** Common food packaging products and sample preparation. (a) Schematic illustration of the structure of the paper, analyzing trends of consumption of plastic packages, investigation of particle contamination from plastic products and cellular cytotoxicity of these particles. (b) Detailed illustrations of the experimental plan. Plastic packaging was boiled to induce nano-sized and micro-sized particle leachates. Leachates were first assessed for size and shape modalities such as DIPA, DLS, FE-SEM, and ATR-FTIR, and filtered to nanometer (nanoplastics) or micrometer (microplastics) categories before staining with iDye Pink. After staining, RAW264.7 murine macrophage cells were treated with water, stained microplastics or nanoplastics on a coverslip within 24-wells plate, before immunofluorescence staining and confocal microscopy were performed to observe specific localization within cellular organelles.

### 3. Results

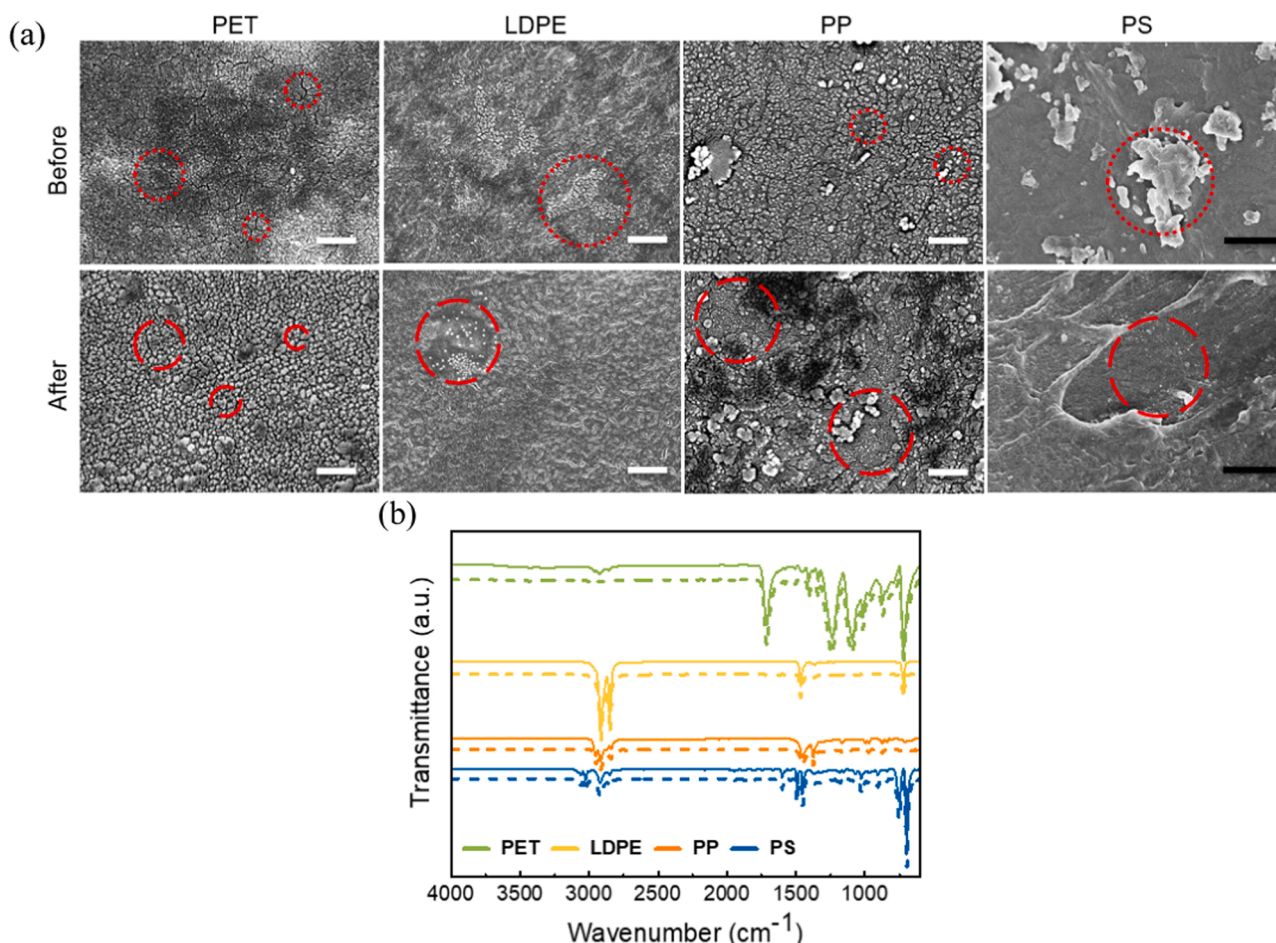
#### 3.1. Erosion of particles from the surface of plastic packaging

Plastics remain one of the cheapest and most accessible and durable materials for packaging applications. Even in paper-based food packaging, a thin layer of plastic is indispensable for waterproofing. In addition, because plastics can adequately tolerate a wide range of temperatures, they are used in high-temperature applications, such as to contain hot beverages and in cooking and microwaving. PET, low-density PE (LDPE), PP, and PS are four polymers predominantly used in both flexible and rigid plastic food packaging (Fig. 1, Figs. S1–5). Therefore, for this study, we collected various food packaging products made of these four materials (Fig. 1c), such as standard disposable rigid plastic cups, (PET, typically used to contain hot beverages), cling wraps, (LDPE, typically used to wrap sandwiches and left on during microwaving), disposable cups and containers, (PP, typically used to contain hot food and soup), and white styrofoam boxes (PS, typically used as takeaway containers for hot foods with gravy). Given their use with hot foods and beverages, these plastics are exposed to temperatures exceeding 90 °C. Furthermore, PP and PET containers are often reused due to their apparent stability (i.e., rigidity and robustness) and their reputation as being microwave-safe, making them convenient for the storing and repeated heating of food. Therefore, we examined the robustness—that is, the absence of leached contaminants—of the

collected plastic packaging products under the aforementioned heating conditions. The collected products were thoroughly cleaned and cut into four squares, each measuring 2.5 cm × 2.5 cm (total surface area: 50 cm<sup>2</sup>).

We designed our experiments such that the actual usage of the aforementioned packaging products in everyday life was replicated (Fig. 2b). Specifically, to simulate their exposure to hot surfaces or foods and beverages, the samples were steeped in deionized (DI) water pre-heated to 95 °C for 10 min in clean glass vials. A comparison of the surface morphologies of the samples via field-emission scanning electron microscopy (FE-SEM) before and after steeping presented noticeable differences (Fig. 3a). Before heating, the PET, LDPE, and PP samples were observed to have a smooth surface. After heating, however, physical defects manifesting as cracks and gaps appeared on the surface. In contrast, before heating, the surface of the PS samples had particles that are inherent byproducts of the PS manufacturing process; these particles were markedly absent after heating. Overall, the results strongly suggest that the surfaces of all four examined plastics had undergone erosion upon steeping.

The characteristic vibrations of the samples before and after heating were recorded through Fourier-transform infrared (FTIR) spectroscopy (solid and dashed lines in Fig. 3b, respectively). The FTIR spectra showed characteristic peaks attributable to PET, LDPE, PP, and PS (Jung et al., 2018) and no observable chemical changes after heating, as indicated by the unchanged wavenumber and transmittance; these



**Fig. 3.** Characterization of samples derived from plastic food packaging products. (a) FE-SEM images showing physical defects (cracks and gaps) that develop during the normal usage of the food packaging products from which the samples are derived; these defects are also visible to the naked eye. The red lines demarcate representative areas on the respective sample's surface that showing marked differences in topographical features. White scale bars: 300 nm. Black scale bars: 2  $\mu$ m. (b) FTIR spectra showing the absence of any chemical changes in the samples after heating. Solid and dashed lines represent the spectra recorded before and after heating, respectively.

results strongly support the hypothesis that the morphological differences observed via FE-SEM were due to heat-induced surface degradation.

### 3.2. Detection and characterization of leached plastics after first usage

The surface erosion of the samples, as evidenced by the FE-SEM observations, strongly suggest the leaching of fragments of the samples into the medium (water) they were steeped in. The size distributions of the particles in the water in which the samples were steeped were characterized via dynamic light scattering (DLS) analysis. Particles that leached from the PET, LDPE, and PS samples were of similar size, whereas those that leached from the PP sample were much larger (Fig. 4a).

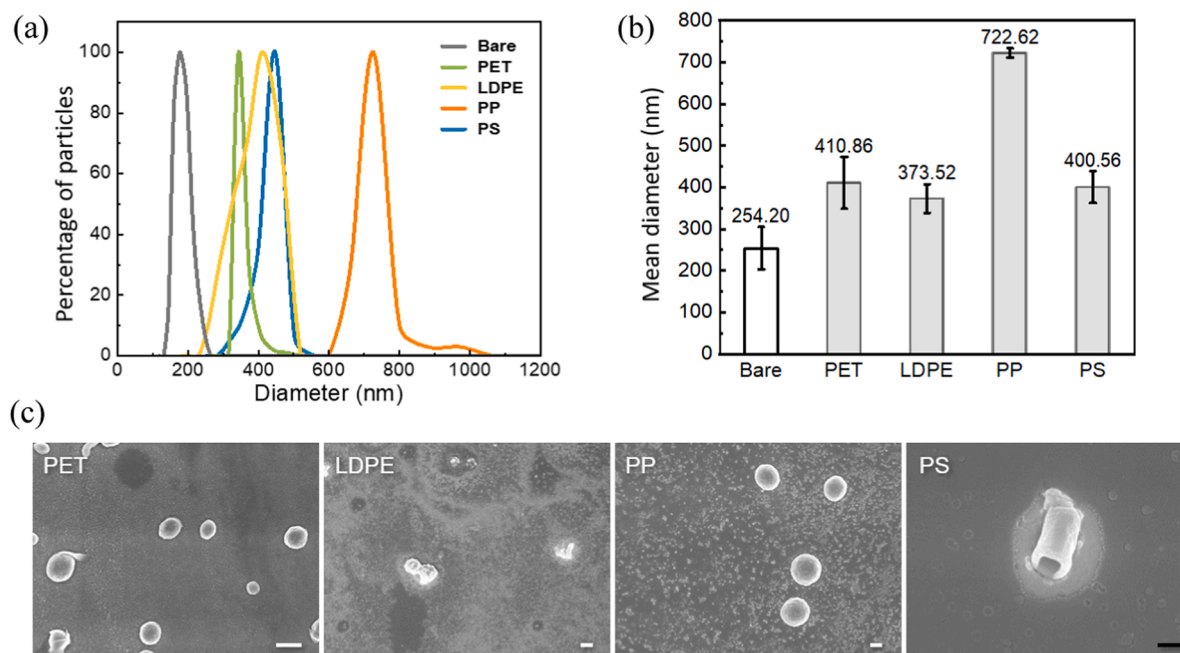
The mean diameters of the particles that leached from the PET, LDPE, PP, and PS samples, calculated based on percentage distribution, were 410.9, 373.5, 722.6, and 400.6 nm, respectively (Fig. 4b), which are significantly larger than a bare control (i.e., DI water in which no samples were steeped; Fig. 4a and b) and thus strongly support the foregoing SEM and FTIR characterizations. Furthermore, positive fluorescent staining observed via confocal laser scanning microscopy (CLSM) confirmed that particles in the water in which fluorescent-labeled (iDye Pink) PET samples were steeped were indeed released from the samples (Fig. S6). SEM and DLS analysis of the post-treatment water dried onto the surface of glass slides showed the presence of well-aligned nanoplastics (Fig. 4c and Fig. S7), which were absent in the bare control water (Fig. S8a). Most PET and PP particles were round, whereas the LDPE and PS particles were of irregular shape. To further verify the origin of the leached particles, the concentration of particles (per milliliter) present in each post-treated water sample was measured through dynamic image particle analysis (DIPA) using FlowCam (Fig. S8b). The bare control water had a particle concentration less than  $1 \times 10^5$  per mL, whereas the water in which the LDPE and PS samples were steeped both had approximately 10 times the particle concentration of the bare control. The corresponding leached particle concentrations in the PET- and PP-steeped water exceeded  $5 \times 10^7$  particles per mL. These large particle concentrations confirmed that the particles

were released from the plastic samples and that they are likely to be nanoparticulate fragments released due to the heat-induced surface erosion of the samples.

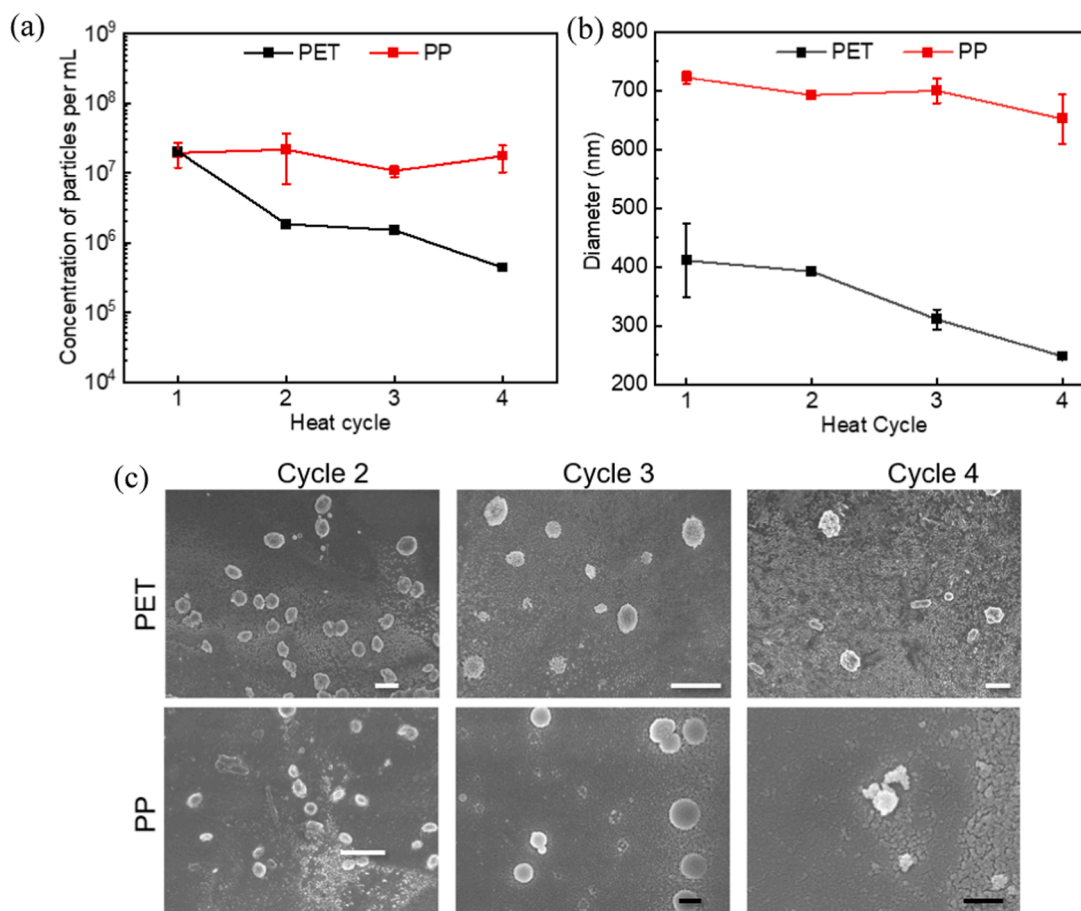
### 3.3. Plastic particles released from reused plastic food containers

PET and PP products are widely used to package or carry beverages and food due to their clarity, rigidity, and convenience and their reputation as being microwave-safe. Hence, PET and PP products tend to be used for food storage in fridges and repeated reheating in microwave ovens, while LDPE and PS are normally single-use plastics. Given the observed erosion of plastics right from their first use, we examined the profiles of the leached plastic nanoplastics following repeated heat treatment. Specifically, to conservatively simulate the reuse of plastic food containers, we subjected the PET and PP samples to the steeping treatment for three additional cycles and analyzed the steeped water after each heat cycle, as described previously.

DIPA revealed the continuous leaching of nanoplastics from both the PET and PP samples (Fig. 5). The PET plastic samples showed a progressive reduction in the concentration of leached nanoplastics over the four steeping cycles, from 50 million particles per mL to 600,000 per mL (Fig. 5a). The PET nanoparticles that leached during the first cycle of heat treatment were generally larger (approximately 400 nm) than those during the subsequent cycles, which were reasonably uniform varying from 400 nm to 200 nm (Fig. 5b). This result suggests that the surface of the PET packaging material is more susceptible to heat treatment and less robust than its inner matrix. In contrast, particle leaching from the PP samples was consistent throughout the four heat cycles (50 million to 40 million particles per mL), indicating a continuous and consistent surface erosion (Fig. 5a). Furthermore, the size of the leached PP nanoparticles progressively decreased from 750 to 620 nm over the four cycles (Fig. 5b). SEM analyses showed that the PET and PP nanoparticles that leached during the second heat cycle were much smaller than those from the first cycle, consistent with the DLS analysis (Fig. 5c).



**Fig. 4.** Characterization of plastics leached from the PET, LDPE, PP, and PS samples. (a) Particle size distribution characterized via the DLS analysis of the water in which the samples were steeped at 95 °C for 10 min (b) Mean diameter of the plastic particles calculated from the particle size distribution. (c) SEM images of the plastic particles leached from the examined packaging products. White scale bars: 1 μm. Black scale bar: 200 nm.



**Fig. 5.** Characterization of particles leached from the PET and PP samples over four heat cycles. (a) Concentration (particles per milliliter) characterized after each cycle treatment, indicating the release of plastics during the repeated use of plastic packaging products. (b) Diameter of particles characterized after each cycle treatment, indicating the release of plastics during the repeated use of plastic packaging products. (c) FE-SEM images of leached particles from PET and PP packaging products dried onto a glass substrate. White scale bars: 2  $\mu$ m. Black scale bars: 300 nm.

### 3.4. Cellular ingestion of plastic micro- and nanoparticles

Macrophages are the first line of defense against ingested pathogens and foreign materials in the mammalian system. They readily detect and remove pathogens and dying cells to prevent infection and maintain general cell function. To investigate the ease of uptake of the leachates by the macrophages, we harvested a significant amount of plastic particles via physical grinding and of the respective samples. Given that all the plastic samples were thermoplastics (Williams and Williams, 1997; Ahmad et al., 2017; Gu et al., 2017; Shubhra et al., 2011; Dai et al., 2020), particles eroded from the surface of these plastic samples either via heat treatment or physical grinding would be chemically indistinguishable. After a series of filtration to obtain particles in the micrometer or nanometer scale, we incubated murine macrophage RAW264.7 cells in vitro with 4  $\mu$ g/mL of the obtained plastic micro- and nanoparticles. Within 3 h of incubation, both the micro- and nanoparticles were internalized by the cells. Nano-PET particles tagged with iDye Pink polymer dye can be observed even 24 h after incubation (Fig. 6a). Confocal microscopy revealed that the nano-LDPE ( $160.5 \pm 1.9$  nm), micro-LDPE ( $1.302 \pm 0.25$   $\mu$ m), micro-PET ( $1.83 \pm 1.01$   $\mu$ m), and micro-general purpose PS (GPPS;  $1.62 \pm 0.80$   $\mu$ m) particles had significantly lower uptake than did the nano-PET particles (Fig. 6a and Fig. S11).

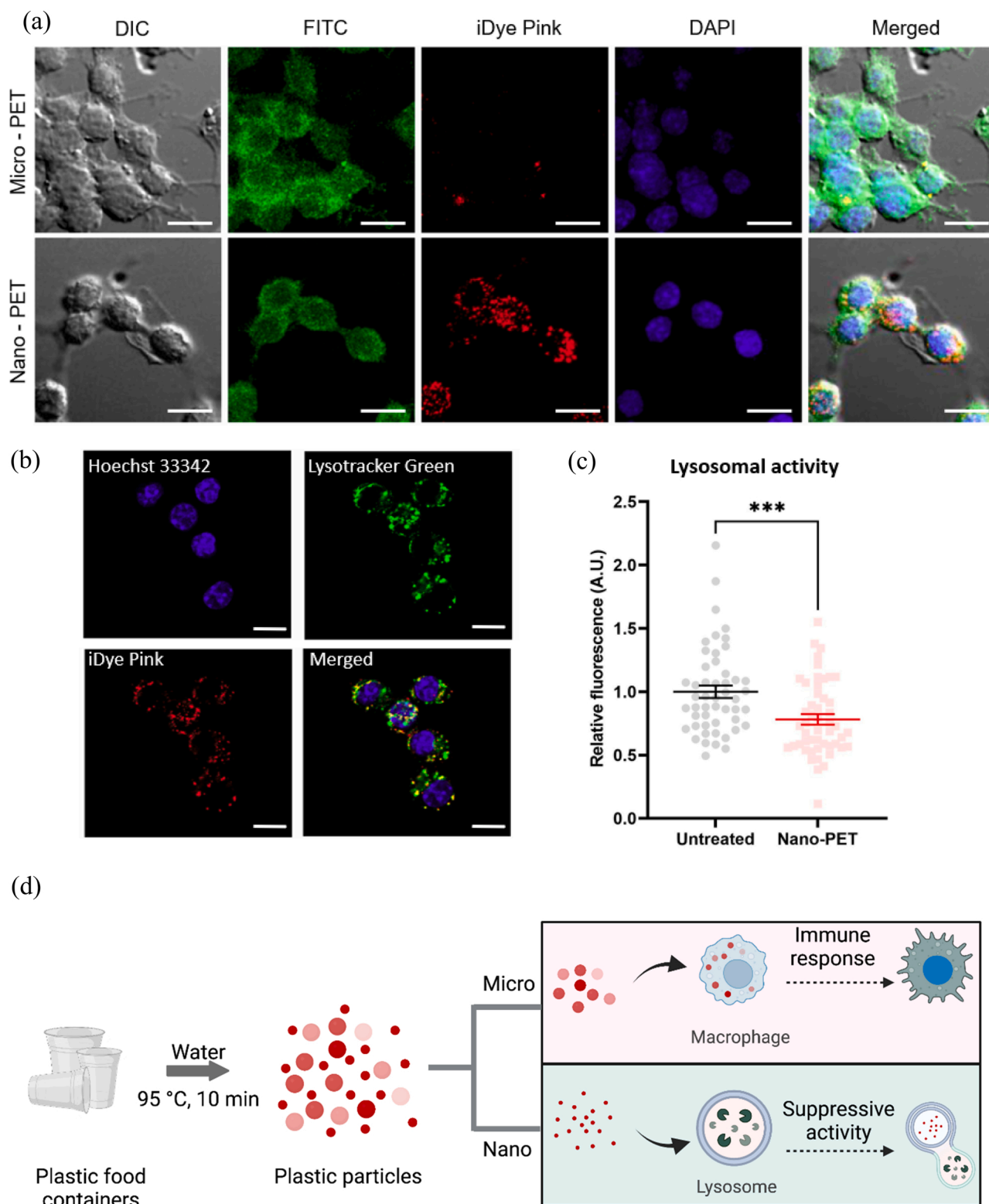
Interestingly, macrophage cells exposed to microplastics exhibited a flattened morphology, which is indicative of immune activation (Somasundaram et al., 2020), whereas cells incubated with nanoparticles were likely to retain a more rounded, dormant appearance (Fig. 6a and

Fig. S11). Furthermore, the ingested nano-PET particles accumulated within the lysosomes and cytosol (Fig. 6b) but not the mitochondria or other organelles (Fig. S9). The uptake of the nano-PET particles significantly suppressed the lysosomal activity (Fig. 6c), which has been associated with compromised cellular degradation processes and enhanced generation of reactive oxygen species (Jin, 2006; Komatsu et al., 2006). Despite prolonged treatment with nano-LDPE, micro-LDPE, micro-PET, and micro-PS particles for up to 40 h, the viability of RAW264.7 cells was not affected (Fig. S11).

Overall, environmentally leached plastic micro- and nanoparticles were readily taken up by systemic macrophages. In the short term, these particles can lead to varying degrees of inflammation, with a negligible effect on cellular survival. However, prolonged disruption of lysosomal activity and cellular homeostasis inevitably leads to cellular damage and tissue degeneration, which are reminiscent of the pathologies observed in lysosomal storage diseases and neurodegenerative proteinopathies (Cortes and La Spada, 2014; Parkinson-Lawrence et al., 2010).

## 4. Discussion

Concerns over the use of plastic products in heat applications have recently been rising, with an increasing number of studies showing the moderate leaching of microplastics into food and beverages, even in everyday use products such as bottled water, salt, and sugar (Cox et al., 2019). Despite microplastics being a plausible global concern, the detrimental environmental and direct health effects of microplastics remain largely unknown.



**Fig. 6.** PET nano-plastic particles were internalized into RAW264.7 macrophage-like murine cell lines. (a) Numerous nano-PET ( $191.6 \pm 0.9$  nm) particles can be found highly internalized in cells, while micro-PET ( $1.85 \pm 1.02$   $\mu$ m) particles were less internalized into the cells after 24 h of incubation. (b) Plastic particles were observed in the lysosomes as fast as 3 h after the initial addition of Nano-PET particles into the culture media. (c) Lysosomal activity was significantly decreased with a P-value of 0.0008 in nano-PET treated cells ( $0.782 \pm 0.04139$ ) compared to untreated cells ( $1.00 \pm 0.0485$ ) after 3 h of incubation. Error bars represent the standard error of the mean (SEM). \* \* \* indicates p-value < 0.001. FITC represents GAPDH, DAPI and Hoechst 33342 represent nucleus, Lysotracker green represents lysosomes, iDye Pink represents PET particles. Scale bars: 10  $\mu$ m. (d) Conclusive mechanism and effects of plastic particles on macrophage cells and lysosome activities. Ingestion of micro-sized plastic particles by macrophages is likely to induce proinflammatory response, while internalization of nano-sized particles inhibits lysosomal functions.

We found that moderate heat application to plastic food packaging products, similar to the temperature range expected in their regular usage, releases plastic micro- and nanoparticles (Fig. 3a). In the experiments, cracking and shearing of the plastic surfaces were evident to the naked eye even before any thermal treatment, with these defects releasing millions of micro- and nanoparticles. This finding contradicts the common belief that newly synthesized plastics have smooth surfaces and are highly resilient to thermal decomposition (Chamas et al., 2020; Ioakeimidis et al., 2016). We argue that surface shearing is probably due to physical erosion rather than chemical alteration, as the effective degradation temperature of plastic polymers is well over a few hundred degrees Celsius (Singh et al., 2019). This argument was supported by our FTIR analysis, which showed no observable chemical changes in the composition of any of the samples after heating. The release of microplastics and nanoplastics, which are of various shapes and sizes, is highly likely to be due to the heat-induced flaking of the cracked plastic surfaces. Furthermore, our findings reveal that the repeated heating of PET and PP packaging results in the consistent leaching of plastic particles (Fig. 5), suggesting that reheating food and beverages in such “reusable” plastic containers is a food safety risk.

The health concerns associated with the excessive ingestion of micro- and nanoplastics are well-grounded. Particles ingested together with food and beverages first enter the gastrointestinal tract and are deposited at the mucosal epithelia of the stomach and the intestines. Most plastics are retained in the gut and eventually excreted by defecation (Schwabl et al., 2019), while particles smaller than 150  $\mu\text{m}$  likely bypass the tight junctions in the intestines and enter systemic circulation (Barboza et al., 2018). Such infiltration into circulation poses several risks, of which the most severe include potential uptake into cells, especially innate immune cells (e.g., macrophages and neutrophils) and the cells of major filtration organs. The absorption of microplastics by these cells results in the release of cytokine factors that promote a pro-inflammatory response. In other cells, microplastic ingestion may cause a reduction in cellular viability. In zebrafish models, plastic particles have been found deposited within the gills, liver, and gut (Deng et al., 2017) and have been widely associated with inflammation, organ failure, and behavioral alterations.

Although the cellular effects of microplastics have been reasonably well studied, not much is known on whether nanoplastics exert similar effects. In this work, we showed for the first time that leached PET nanoparticles are readily taken up, likely through phagocytosis, by murine macrophages in as little as 2 h of incubation (Fig. 6a). Fluorescence from labeled PET nanoparticles were observed to colocalize with the lysosomal dye, suggesting that the nano-PET particles tend to reside within the phagosomes or phagolysosomes in the cells.

Lysosomes are critical for cellular homeostasis and metabolic regulation (Lim and Zoncu, 2016), and their functions are dependent on the activity of lysosomal proteins. Interactions between cellular proteins and microplastics change the secondary structure of the proteins, rendering them dysfunctional (Holl  czki, 2020; Holl  czki and Gehrke, 2019). A coalescence effect is possible, whereby the nanoplastics act as a scaffold for the accumulation of proteins, resulting in protein mislocalization and dysfunction (Gopinath et al., 2019). Supporting this theory and the argument that microplastics are toxic to living organisms, our experiments revealed a decrease in lysosomal activity, more specifically hydrolytic enzymes activity, with PET uptake and accumulation in RAW264.7 cells (Fig. 6d). Correspondingly, these findings suggest that PET can interact directly or indirectly with key lysosomal components, such as hydrolytic enzymes or V-ATPase proton pumps, to inhibit the capability of the lysosome to digest autophagic and phagocytotic debris. Our findings also matched the results from the previous studies in showing that plastic particles can cause other substantial lysosomal defects in marine animals through altering lysosomal pH (Tan et al., 2020), inducing lysosomal membrane destabilization (von Moos et al., 2012), and aberrant aggregation within the lysosomes (Brandts et al., 2020). In rare instances, the particles may escape into the cytoplasm and

disrupt other organelle functions, such as altering mitochondrial membrane potential and inhibiting plasma membrane ATP-binding cassette (ABC) transporter activity (Wu et al., 2019). In many papers, the cellular interaction and uptake of plastic particles have also been associated with the release of pro-inflammatory cytokines by immune cells (Hwang et al., 2020; Hirt and Body-Malapel, 2020), reminiscent of the innate immune response triggered to target and remove foreign materials in the body. The long-term effects of ingested nanoplastics on cellular viability and the normal physiological function of macrophages remains unknown, warranting studies focused on the biological effects of nanoplastics and their downstream effects on intracellular biochemical pathways. Whether the addition of additives would alter plastic properties and whether such alterations would affect how cells ingest plastic are unclear (Rahman and Saleh, 2012). Apart from sequestering toxins (Wang et al., 2018; Verla et al., 2019), microplastics are prone to homoaggregation through van der Waals forces (Tallec et al., 2019; Wang et al., 2021), which may obstruct normal cellular or tissue functions. Relatively small microplastics may be taken up by non-specific or receptor-mediated cells, particularly phagocytes such as macrophages (Behzadi et al., 2017).

Recent studies are also beginning to shed light on the interaction of microplastics with the proteins from the surrounding interstitial environment. It is possible that plastic particles and the interstitial proteins may form protein corona that facilitates uptake of the plastic particles through receptor mediated processes. Transferrin in the protein corona found around nanoceria nanoparticles was previously reported interact with transferrin receptor to modulate clathrin-mediated endocytosis (Mazzolini et al., 2016). Similarly, polystyrene (PS) particles were reported to form corona in fetal bovine serum (FBS), internalizing into cells, and thereafter impair lysosomal activity (Tan et al., 2020). The size of the nanoparticles also influences composition of the protein corona; small plastic particles have been known to favor the formation of soft corona, while large plastic particles favor the formation of hard corona (Kihara et al., 2019). Relatively small micro- or nanoplastics may be uptaken either through phagocytosis or receptor-mediated processes, particularly by phagocytes such as macrophages (Aguilar-Guzm  n et al., 2022). Further research is necessary to investigate the changes to the corona composition, and the potential role of its uptake into specific cells.

## 5. Conclusion

In this study, we observed that millions of micro- and nano- plastic particles can be released from plastic food packaging after being exposed to high temperatures (food heated or packaging hot beverages). Our discoveries further revealed that repeated high-heat treatment of plastic food containers, especially PP and PET, brings consistent leaching of microplastics, thus contaminating the food packaged and increasing food safety risk. Microplastics released from such packaging were presented to be immediately engulfed by RAW264.7 mouse macrophages in vitro and suppressed cellular viability. Our findings clarify the immediate negative implications of the use of plastics in food packaging. Overall, demands of plastic products and packaging extremely rise during the COVID-19 pandemic, our findings further emphasize the need for an alternative material that can replace plastics to palliate plastic threatens.

## Environmental Implication

Plastic contamination aroused great attention of the society due to surging numbers of wildlife dying from plastic pollution. Especially during COVID-19 pandemic, the escalating use of hot food take-away containers directly threatens human health and the leached micro/nanoplastics may be uptaken by human body with long term toxic effects. Most researchers have focused on plastic particles accumulating in food chain through marine organism or mammals. This research

introduced higher sensitive analysis methods with great resolution and revealed plastic particles released from food packagings and direct harmful effects on immune cells.

### CRedit authorship contribution statement

**Jingyu Deng:** Conceptualization, Methodology, Investigation, Writing - original draft, Writing - review & editing. **Mohammed Shahrudin Bin Ibrahim:** Conceptualization, Methodology, Investigation, Writing - original draft, Writing - review & editing. **Li Yang Tan:** Conceptualization, Methodology, Investigation, Writing - original draft, Writing - review & editing. **Xin Yi Yeo:** Investigation, Resources, Writing - review & editing. **Yong An Lee:** Investigation, Writing - review & editing. **Sung Jin Park:** Investigation, Writing - review & editing. **Torsten Wüstefeld:** Investigation, Writing - review & editing. **June-Woo Park:** Investigation, Writing - review & editing. **Sangyong Jung:** Conceptualization, Methodology, Writing - review & editing, Supervision. **Nam-Joon Cho:** Conceptualization, Methodology, Writing - review & editing, Supervision.

### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

### Acknowledgements

This work was supported by the AcRF Tier 1 granted by Ministry of Education (MOE), Singapore (grant numbers TIER1-2020-T1-002-032). This work was also supported by the China-Singapore International Joint Research Institute (CSIJRI). Besides, this work was also supported by A\*STAR - Joint Council Project Grants, Singapore (Grant No. BMSI/15-800003-SBIC-OOE).

### Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.jhazmat.2022.128980](https://doi.org/10.1016/j.jhazmat.2022.128980).

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